FEMALE PREFERENCE FOR MALE SWORD EDGE IN HYBRID *XIPHOPHORUS BIRCHMANNI*-TYPE SWORDTAILS

An Undergraduate Research Scholars Thesis

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ABSTRACT

Female Preference for Male Sword Edge Pigmentation in Hybrid *Xiphophorus birchmanni*-type Swordtails

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Female preference for male traits can drive the asymmetric introgression of male secondary sexual characteristics. In the Rio Calnali, *Xiphophorus birchmanni* and *Xiphophorus malinche* have hybridized to create two distinct genetic clusters within the same area. The *X. malinche* cluster shows a small amount of introgression from *X. birchmanni*, but the *X. birchmanni* cluster is genetically 75% *X. birchmanni* and 25% *X. malinche*. Females of an allopatric population of *X. birchmanni* demonstrate a preference against the male sword ornament on the caudal fin while females from an allopatric population of *X. malinche* show no preference for this trait. The *X. birchmanni* cluster from the Rio Calnali shows introgression of the pigmentation on the lower caudal fin but not of the sword extension. Using dichotomous choice tests and computer animations made with the Anyfish software platform, female hybrids were tested for preference for parental species, *X. birchmanni* versus *X. birchmanni* with an X. *malinche* sword, and hybrid *X. birchmanni* versus *X. birchmanni* with an added sword edge pigmentation. Low sample size of *X. birchmanni*-type females prevented accurate conclusions, but *X. malinche*-type females showed no preference for the three stimuli. Further research must be conducted to increase sample size, confirm lack of *X. malinche*-type preference, and ascertain differences in personality between clusters

CHAPTER I

INTRODUCTION

Consequences of hybridization

Genetic exchange between divergent species can facilitate the introgression of adaptive traits into new populations, but the consequences of sexual selection on this exchange are not fully known. Hybridization is an important force in evolution, and sexual selection via mate choice could be an important factor in the genetic exchange between species. Further, female mating preference could favor traits not found within their own species which could facilitate hybridization and asymmetric introgression of the preferred trait across the hybrid zone into the parental population lacking the trait.[1-3]

An example of asymmetric introgression

A well-known example of this would be the Manakins, Central American birds known for their colorful plumage. In certain areas where hybrid zones have arisen, the golden collar plumage of *Manacus vitellinus* has introgressed into the white-collared *Manacus candei* population. [18-20] This introgression appears to be driven by the female preference for golden-collars and the more aggressive behaviors associated with golden-collared males. This system-wide preference coupled with the viability of hybrids and low cost of hybridization facilitates the gene flow of golden-collars from *M. vitellinus* to *M. candei* through the hybrid zone of lemon-collared males which more resemble the golden-collared parental species in appearance and behavior. [1, 2][2, 3]

Origin of sword and sword preference

The genus *Xiphophorus* ranges from northern Mexico through Central America. These live-bearing fish inhabit shallow areas of slow-moving water where they consume algae and small invertebrates. *Xiphophorus* itself is divided into two groups: swordtails and platyfish. The most notable distinction between the two are that platyfish have reverted to the ancestral state of swordlessness while the males in some species of swordtails retain an extension of the lower caudal rays developed at the base of the genus. [3] Although the sword is only found within the swordtail lineage, the preference for the sword exists in both the swordtails and the platyfish indicating that the preference is ancestral. [4-11]

According to phylogenetic data the ability to produce a sword was developed in the common ancestor of *Xiphophorus* with most swordtails retaining this trait and platyfish reverting to the swordless state. [3] Although they may possess other sword traits such as pigmentation, platyfish lack the extension of the sword, but platyfish females demonstrate a preference for artificially sworded conspecific males [6, 7] Further, females in an outgroup, the genus *Priapella*, also prefer sworded males indicating that the sword preference is ancestral and arose before the appearance of the sword in *Xiphophorus*. [4-7] In contrast to *Priapella* and the platyfish, some species of swordtail have lost this preference for the sword such as in *X. malinche* or reversed preference to sword aversion such as in *X. birchmanni*.

Explanations for preference and appearance of sword

Preference for the sword and the sword's development appears to stem from the females' bias for larger size. The sword exploits this pre-existing female bias in a metabolically inexpensive way.

When given a choice between animated stimuli manipulated only for the trait in question, increasing the size of the swordless male to match the apparent size of the sworded male causes females to prefer enlarged swordless male and sworded male roughly the same. [13] The female size bias for males is present in other genera of poeciliids where fins other than the caudal fin are modified. Examples of this include the elongation of the gonopodium in Priapella and the extension of the dorsal fin in Poecilia. These modifications of male fins could indicate that these are evolutionary responses to a perceptual bias for larger male size in poecilliid females. The variety of size-enhancing traits may represent a permissiveness of female preference for traits which increase apparent size without increasing actual body size. [9, 13]

X. birchmanni and *X. malinche* system

Xiphophorus malinche and *Xiphophorus birchmanni* are Northern swordtails with partially overlapping ranges in the Southern Rio Pánuco drainage of Central Mexico. [7, 8] *X. malinche* inhabits higher elevations and has a small and shallow body. The males possess a sword, an extension of the lower caudal fin rays, with a pigmented sword edge. *X. birchmanni* inhabits lowland areas, is larger and deeper-bodied than *X. malinche*, lacks the sword and sword edge coloration, and has a fatty cephalic hump.[7, 8] As a whole, swordtails are characterized by large amounts of reticulate evolution generating new species from hybridization. Further, female swordtails favor mating with hybrids allowing the mechanisms and consequences of hybridization to be studied. [4, 8, 14]

Preferences for the sword in X. malinche and X. birchmanni

Mate-choice experiments using *X. birchmanni* and *X. malinche* have shown that these species demonstrate reduction or reversal of sword preference from the ancestral state. *X. malinche* males possess swords, but when *X. malinche* females are given the choice between sworded vs unsworded male animations, they show indifference to the presence of the sword. In contrast, where *X. birchmanni* males lack the sword and sword edge, *X. birchmanni* females demonstrate a reversal of the ancestral preference by showing antipathy towards the sword. [2-9]

Reasons for reduction or reversal

The reduction and reversal of female preference for the sword in *X. birchmanni* and in the hybrids could be a response to predation by *Astyanax mexicanus* which resides in the same locality as *X. birchmanni. A. mexicanus,* a non-lethal fin-nipping predator, preferentially associates with sworded males. The association cost for females courting sworded males could have driven the reversal of preference currently seen in *X. birchmanni*. [7, 9, 11, 12][8, 10, 12, 15]

Hybrids of X. malinche and X. birchmanni

At intermediate elevations in all known streams where the ranges of *X. birchmanni* and *X. malinche* overlap, hybrid zones have formed. In the Rio Calnali hybrid zone two distinct hybrid clusters have developed. Despite genomic evidence of extensive hybridization, the two clusters remain genetically distinct. [16] Based on next-generation sequencing, *X. malinche*-cluster hybrids are mostly *X. malinche* with some introgression from *X. birchmanni*, but the *X.*

birchmanni-cluster is about 75% *X. birchmanni* and 25% *X. malinche*. [15] Although the preferences of the parental species have been studied, only preliminary data has been collected on the preferences of this population of hybrids.

Reasons for the hybrid zone and continued hybridization

Fisher et al. identified humic acid as a potential factor in disrupting olfactory communication which could have facilitated the creation of this hybrid zone. When *X. birchmanni* females were exposed to water treated with humic acid vs water without the contaminant, the females lost the ability to discern olfactory cues from conspecific and heterospecific males. [13] In 1988, surveys of Rio Calnali yielded no evidence of hybridization, but in a survey in 1997, hybrids were identified. Genetic analysis places the initial hybridization in Rio Calnali to be 35 generations ago. [8, 14] The genetic and survey data indicates that the hybridization event is relatively recent. During this time period, Rio Calnali has been exposed to increasing human disturbance from sewage and agricultural runoff which likely increased the levels of humic acid in the river. [13] The hybridization of *X. birchmanni* and *X. malinche* is widespread with most individuals being hybrids and few pure parental individuals. The lack of F_1 hybrids may indicate that the disturbance which caused the hybridization may no longer be affecting some populations. [15, 16] Additionally, unpublished data shows that the two genetic clusters of Rio Calnali hybrids are mating assortatively. (Schumer and Squire, unpublished data)

Asymmetric introgression in Rio Calnali

Preliminary data has shown evidence of asymmetric introgression of individual traits of the sword complex. [8] As neither parental species demonstrates a preference for the sword, one

might expect the absence of the sword complex to introgress. While the sword extension has not introgressed fully into the *X. birchmanni*-cluster population, the sword edge coloration appears at higher rates than expected. The *X. birchmanni*-cluster possesses a very small sword extension, but even within *X. malinche*-cluster populations, the swords are reduced and appear intermediate to the swords of the two parental species. [11, 12] The preferences of the hybrid females for the sword edge is unknown, but the preference or possible introgression of preference from a parental species could be a factor in the asymmetric introgression of the male traits.

Testing for asymmetric introgression

One explanation for the introgression of the sword edge pigmentation into the *X. birchmanni* genomic background is sexual selection via female mate preference. Hybrid female preference for this trait can be measured to determine if female choice is responsible for the observed pattern of introgression. By determining the preference of hybrid *X. birchmanni*-type cluster females compared to pure *X. birchmanni* females, we can determine if the hybrid population demonstrates a different preference from the parental population and if the male trait and the female preference have introgressed together. [7] Using the anyFish software, animated males representative of the hybrid population will be created to test female *X. birchmanni*-cluster preference for the asymmetrically introgressing sword edge pigmentation. These animations can be manipulated for sword edge pigmentation while controlling other variables. Additionally, baseline preferences will be gauged by testing the *X. birchmanni*-cluster females' preferences for *X. birchmanni* versus *X. malinche* and sworded *X. birchmanni* versus unsworded *X. birchmanni*.

CHAPTER II

METHODS

Animal collection and housing

All hybrid females tested were collected in June of 2015 from the Rio Calnali in Calnali, Hidalgo, Mexico, using baited minnow traps. The pure *X. birchmanni* used were born and reared in the lab from wild females from the Rio Coacuilco captured in June of 2015. All fish were housed in single-sex colonies in flow-through aquaria located at Texas A&M University. Fish were maintained on a 12:12H light:dark cycle at 24°C and fed Repashy Superfoods gel food twice daily.

Test of visual preference

The females' visual preferences were tested using video playback of computer-animated males. Testing took place in 51x28x33cm tanks which were opaque on all but the short sides. LCD computer monitors connected to a software which automates the beginning and end of the video playback were placed on the short ends of the tanks.

Twenty minutes prior to the beginning of the trials, females were introduced into the test tanks. After the initial acclimation period, the computer monitors displayed the two video playbacks for five minutes followed by a five minute black screen. To control for side bias, videos were then displayed on the opposite screens for the next five minutes followed by another five minutes without playback.

The tanks were virtually divided into three zones, and the females' preferences were gauged by time spent in the zones nearest the monitors with the central zone being neutral. Association

times were recorded by a Biobserve Viewer system which detects the position of the fish, but in the event that the system failed to accurately track the fish, videos were scored manually.

The first set of trials comprised of two preference tests: *X. birchmanni* versus *X. malinche* and sworded versus swordless *X. birchmanni.* In the second set of trials the females were presented *X. birchmanni* animations with absent and augmented sword edge pigmentation to test female preference for this caudal fin coloration.[11]

Multiplex Shotgun Genotyping to assign genetic cluster

After the initial trials, the females which were all marked with elastomer tags (Northwest Marine Technologies) for identification were assigned to a genetic cluster using multiplexed shotgun genotyping (MSG). DNA was extracted from fin clips using the Agencourt bead-based purification method (Beckman Coulter Inc. Brea, CA) following manufacturer's instructions with slight modifications. The fin clips were incubated overnight in a shaking incubator (100 rpm) at 55°C in 94 µl of lysis buffer with 3.5 µl of 40 mg/mL proteinase K and 2.5 DTT, followed by bead binding and purification. Genomic DNA was quantified using a Typhoon 9380 (Amersham Biosciences, Pittsburgh, PA) and evaluated for purity using a Nanodrop 1000 (Thermo Scientific, Wilmington, DE); samples were diluted to 10 ng/µl.

MSG libraries were made as described in Andolfatto et al. 2011.[18] 50 ng of DNA was digested with MseI, and barcodes were ligated to each sample. Five µl of sodium acetate and 50 µl of isopropanol were added to each sample and the samples were pooled and precipitated overnight at -20°C. Then samples were extracted and resuspended in TE (pH 8.0) and purified through a phenol-chloroform extraction and Agencourt beat purification. The pooled samples were run on a 2% agarose gel and fragments between 250-500 bp were selected and purified. Two ng of each

pooled sample was amplified for 14-16 PCR cycles with custom indexed primers. Due to multiplexing with other libraries, samples were sequenced on four Illumina HiSeq 2000 lanes with v3 chemistry.[16]

Creating fish animations

Animations of the male fish were created in the anyFish software. Mature males were digitally photographed for morphometric analysis, and forty landmarks were placed on the photographs using the tpsDig2 software. This landmark data was then used to compute the concensus (population mean) morphology using tpsRelw. To create a skin texture for the animation, a single image of an average male was warped using tpsTransformer. Using the generated concensus file from tpsRelw was used in anyFish to generate a model representative of the mean male from the focal population.

To create the fin textures, the fins from an image of a representative male were overlayed onto guides using Adobe Photoshop CS6. To test for female preference for the caudal fin sword edge pigmentation, an alternate version of the caudal fin was created by using Photoshop to apply black pigment representative of the population mean to the fin.

The anyFish program was then used to create a courting animation for the model. The animation, based on videos of males courting in the wild, consisted of a loop of the male swimming to the middle of the screen, performing the courting behavior, and swimming off screen. The animation is repeated in reverse to return to the beginning, and the animation was looped continuously. Care was taken to match the speed and size of the animation to natural males.[20]

Statistical analysis

To analyze the results of the visual preference test, the relative association time with each cue was measured and the general linear model was used to determine significant preferences at the population level. “Time of day” and “holding tank” were designated as random variables.

CHAPTER III

RESULTS

Results of visual trials

X. birchmanni versus X. malinche

Female *X. birchmanni*-type and *X. malinche*-type hybrid females were tested for association time in dichotomous choice tests with stimuli of male *X. birchmanni* versus *X. malinche*. Values reported are the total value of the association time for each stimuli across two trials which control for order presented and side biases.

Using a paired T-test, *X. birchmanni*-type females were found to have a mean association time for *X. malinche* of 224.261 s and 242.468 s for *X. birchmanni* (mean difference= -18.208 s; degrees of freedom= 12; t-statistic= -0.431; p=0.337). *X. maliche*-type females were found to have a mean association time of 219.892 s for *X. malinche* and 245.144 s for *X. birchmanni* (mean difference=-12.428 s; degrees of freedom=36; t-statistic=-0.779; p=0.221).

Figure 1: Female Association Time for *X. birchmanni*-type females in *X. malinche* vs. *X. birchmanni* Choice Trials

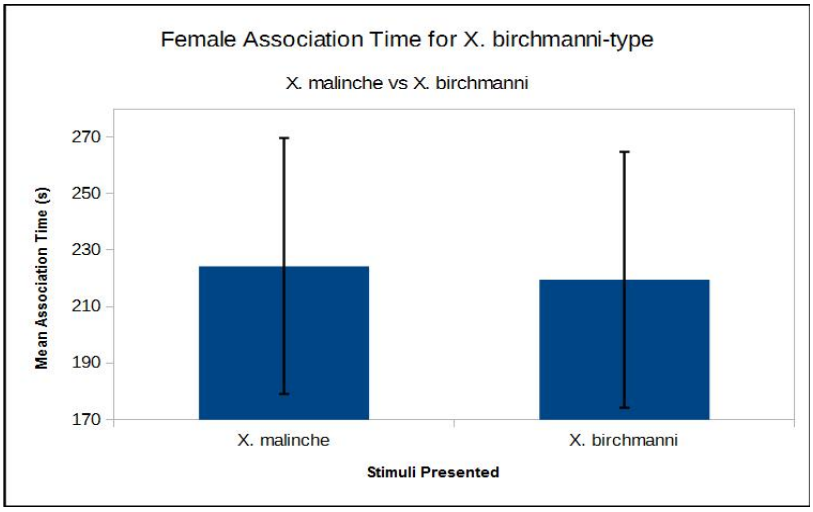
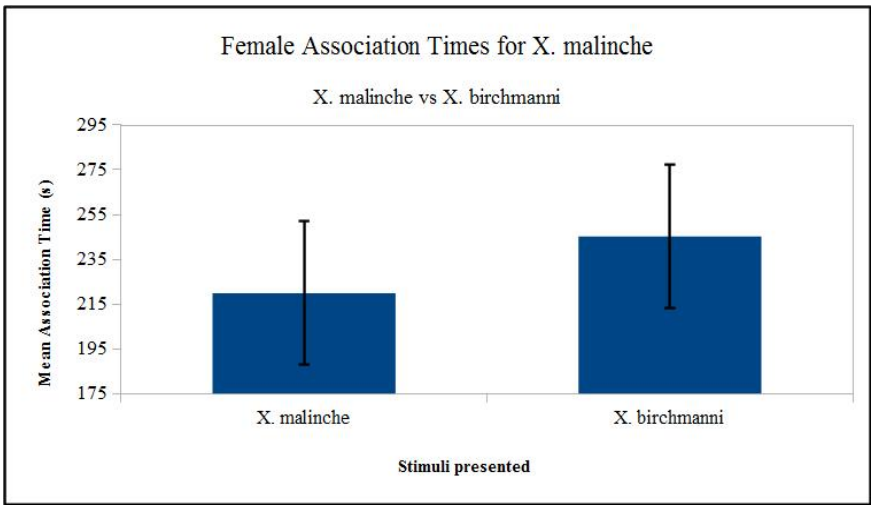


Figure 2: Female Association Time for *X. malinche*-type females in *X. malinche* vs. *X. birchmanni* Choice Trials



X. birchmanni versus Sworded X. birchmanni

Female *X. birchmanni*-type and *X. malinche*-type hybrid females were tested for association time in a dichotomous choice test with animated stimuli of male *X. birchmanni* versus *X. birchmanni* modified to have a sword. Values reported are the total value of the association time for each stimuli across two trials which control for order presented and side biases.

Female *X. birchmanni*-type females were found to have a mean association time of 203.543 s for *X. birchmanni* and 299.852 s for sworded *X. birchmanni* (mean difference= -96.308; degrees of freedom= 9; t-statistic=-3.358; p=0.051). Female *X. malinche*-type females were found to have a mean association time of 214.274 s for *X. birchmanni* and 229.402 s for sworded *X. birchmanni* (mean difference=-15.128 s; degrees of freedom=40; t-statistic=-0.503; p=0.309).

Figure 3: Female Association Times for *X. birchmanni*-type females in *X. birchmanni* vs. Sworded *X. birchmanni* Choice Trials

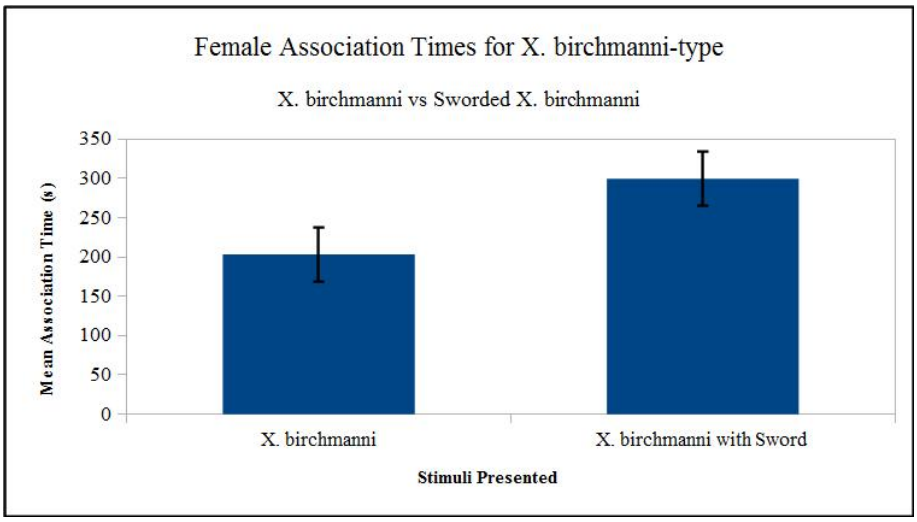
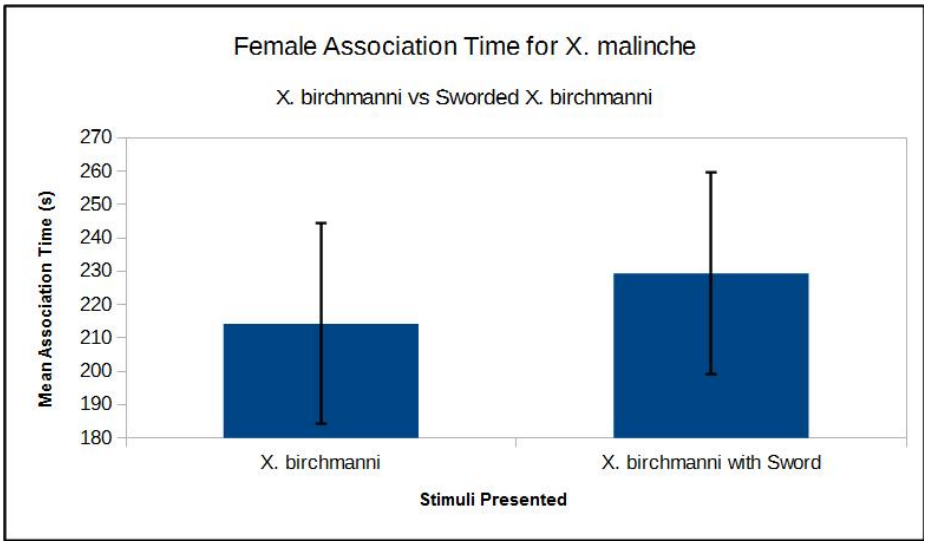


Figure 4: Female Association Times for *X. malinche*-type females in *X. birchmanni* vs. Sworded *X. birchmanni* Choice Trials



X. birchmanni-type hybrid versus X. birchmanni-type hybrid with sword edge

Female *X. birchmanni*-type and *X. malinche*-type hybrid females were tested for association time in a dichotomous choice test with animated stimuli of male *X. birchmanni* versus *X. birchmanni*

with an augmented sword edge pigmentation. Values reported are the total value of the association time for each stimuli across two trials which control for order presented and side biases.

X. birchmanni-type females were found to have a mean association time of 231.238 s for natural *X. birchmanni* and 310.938 s for the augmented *X. birchmanni* (mean difference=-79.700 s; degrees of freedom=5; t-statistic=-1.781; p=0.067). *X. malinche*-type females were found to have a mean association time of 219.721 s for natural *X. birchmanni* and 222.538 s for the augmented *X. birchmanni* (mean difference=-2.817 s; degrees of freedom=16; t-statistic=-0.78; p=0.469).

Figure 5: Female Association Time for *X. birchmanni*-type females in *X. birchmanni*-type vs. *X. birchmanni*-type with Sword Edge Choice Trials

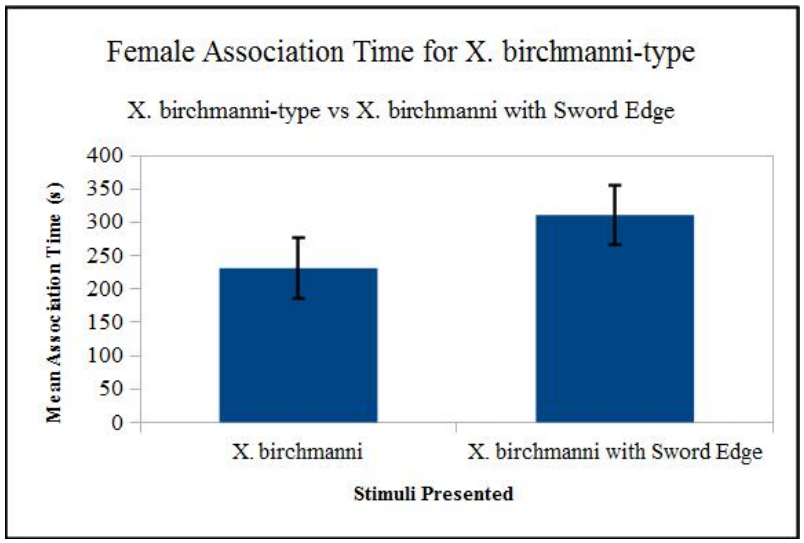
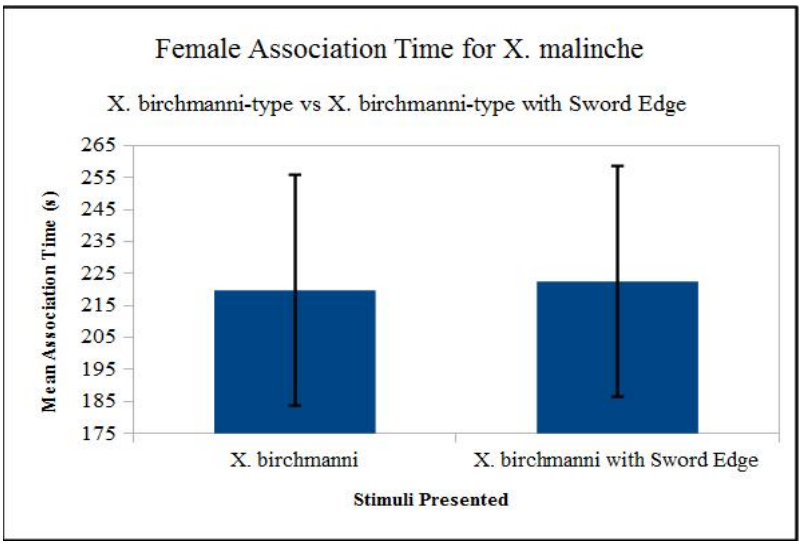


Figure 6: Female Association Time for *X. malinche*-type females in *X. birchmanni*-type vs. *X. birchmanni*-type with Sword Edge Choice Trials



CHAPTER IV

DISCUSSION

X. birchmanni-type female preferences and low sample size

Although the preference of *X. birchmanni*-type females was the focus of this research, conclusions are difficult to make due to the low sample size. After genotyping, individuals were identified by cluster, but *X. malinche*-type females greatly outnumbered *X. birchmanni*-type females. Further, due to delays in making the *X. birchmanni*-type versus *X. birchmanni*-type with sword edge animations, many females died before the third set of trials, further reducing *X. birchmanni*-type sample size.

X. malinche-type hybrids preliminarily show no preference

X. malinche-type females showed no preference in any of the three trials, and, in contrast with the sample size issues with *X. birchmanni*-type females, the sample sizes were considerable. Proving that a population shows no preference can be much more difficult than proving a preference for or against. As preference can be influence by many factors such as deficiencies in the stimulus, lighting, or testing environment, the *X. malinche*-type females must be tested against controls such as platyfish animations or males modified to remove the lower caudal fin. [10, 20]

Personality differences between clusters

Notably, the between-cluster differences in mean association times could indicate a difference in the overall boldness of each cluster. Previous research has identified that pure *X. birchmanni*

tends to be more bold than pure *X. malinche* when using "latency to leave shelter" to measure boldness. From the mean association times, *X. birchmanni*-type females tend to have a much higher mean difference than *X. malinche*-type females which could indicate that the *X. birchmanni*-type females are spending more time associating with the stimulus while *X. malinche*-type females tend to stay near the shelter. A preliminary review of the trial recordings corroborates this, as the *X. malinche*-type females often circled the shelter in the central zone while *X. birchmanni*-type females would dart between association zones and pay little attention to the shelter. [22,23]

Future directions

To remedy the low sample size of *X. birchmanni*-type females, more individuals from Rio Calnali have been captured, and upon cursory visual analysis, a higher percentage seem to be *X. birchmanni*-type. To speed the identification of new individuals in the future, a visual method of identification is being developed and correlated to genotypic data. These new females will be tested for preference in all three trials which will increase the sample size. Additionally, pure *X. birchmanni* will be trialed to find out if any preferences found are innate preferences in the most closely related parental species or if the preference is introgressing from *X. malinche*.

X. malinche-type females, which showed no preference, will be trialed using control animations such as a platyfish or males with the lower caudal fin removed. These controls fully lack the sword complex allowing a better analysis of the *X. malinche*-type lack of preference as related to the sword edge pigmentation. [10, 22]

Finally, a quantitative analysis of boldness based on the latency to emerge from shelter must be completed. A quantitative analysis of hybrid boldness can show differences in cluster personality and could indicate differences in personality between the parentals and the hybrids. As noted in Johnson et. al. 2015, *X. birchmanni* had a lower latency to emerge from shelter, but in the event of a simulated predator attack, they had a lower tendency to "fast-start" and swim away. *X. malinche* tended to have longer latency to emerge from shelter but a higher tendency to "fast-start" when attacked by a simulated predator. Introgression of these characteristics from parental species could create hybrids with different boldness. Quantitative analysis of the latency to emerge from shelter from data collected in this research and future trials of "fast-start" behavior will be necessary to determine hybrid boldness and analyze possible introgression of boldness characteristics in each hybrid cluster. [22, 23]

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